REVIEW ARTICLE

The regulation of tyrosine kinase signalling pathways by growth factor and G-protein-coupled receptors

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INTRODUCTION

The regulation of cellular growth and division by extracellular signals is now a major field of investigation. Within this area of study considerable attention has focused upon the intracellular signalling pathways which initiate early events associated with mitogenesis. These include systems in which specific kinases are activated following the generation of second messengers, for example diacylglycerol (DAG)/protein kinase C (PKC) and the cyclic AMP/cyclic AMP-dependent protein kinase (PKA) pathways.

Recently, major advances have been achieved in the elucidation of cellular signalling pathways which involve the activation of proteins or enzymes by phosphorylation upon tyrosine residues. In this type of pathway cellular signals are generated through a series of protein–protein interactions and a cascade of phosphorylation events rather than by the immediate generation of a second messenger. Historically this type of pathway has been associated with growth factor receptors which display intrinsic tyrosine kinase activity [1]; however, a number of G-protein-coupled receptors have also been found to activate tyrosine kinase pathways. Concentrating on early signalling events, this review will outline the regulation and function of the major tyrosine kinase pathways activated in response to both classes of agonist and place these in the context of other well-established cell signalling pathways.

GROWTH FACTOR INITIATION OF THE TYROSINE KINASE CASCADE AND RECEPTOR–EFFECTOR INTERACTIONS

Growth factor receptors are prototypic members of a family of receptors which are characterized by an extracellular binding domain, a single transmembrane portion and a large intracellular catalytic domain [1]. For these receptor types the key event following growth factor stimulation is autophosphorylation within the intracellular domain accompanied by dimerization of the receptor [1,2]. Autophosphorylation of the platelet-derived growth factor (PDGF) receptor occurs within a number of regions including the kinase insert domain, while for others which lack a kinase insert domain, for example the epidermal growth factor (EGF) receptor, tyrosine residues in the C-terminal tail are major sites of phosphorylation. Receptors within this category are thus designated receptor tyrosine kinases.

Receptor tyrosine phosphorylation promotes interaction of the receptor with a number of target proteins or enzymes. Examples include phosphoinositide-specific phospholipase Cγ (PLCγ) [3,4], the 85 kDa subunit of phosphatidylinositol 3-kinase (PI 3-kinase) (p85) [2], GTPase-activating protein (GAP) [5], growth-factor-receptor binding protein 2 (Grb2) [6,7], the phosphotyrosine phosphatase SHPTP-2 [8] and members of the non-receptor Src family of tyrosine kinases [9,10] (Figure 1 and

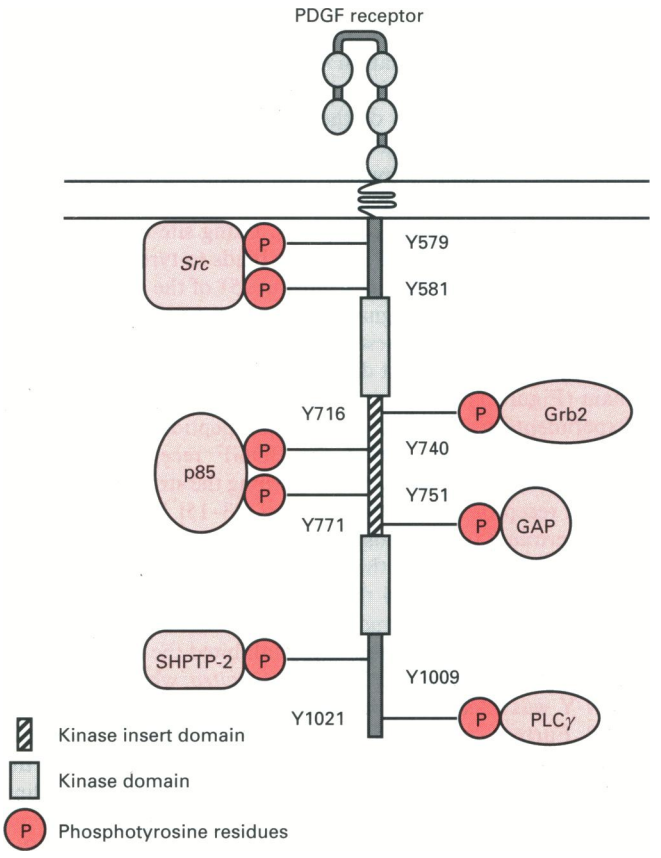

Figure 1 Schematic diagram representing the phosphotyrosine residues implicated in the binding of the human PDGF β receptor to downstream signalling proteins

See refs. [1–15].

Abbreviations used: DAG, diacylglycerol; EGF, epidermal growth factor; FAK, focal adhesion kinase; FGF, fibroblast growth factor; fMLP, formyl-Met-Leu-Phe; FRAP, rapamycin-associated protein; GAP, GTPase-activating protein; G-LRA, G-protein-linked receptor agonist; JAK, Janus kinase; IL, interleukin; IFN, interferon; IRS-1, insulin receptor substrate 1; ISGF-3, IFN-stimulated gene factor 3 complex; Grb, growth-factor-receptor binding protein; LPA, lysophosphatidic acid; MAP, mitogen-activated protein; mSos, mammalian Son of sevenless; NFκB, nuclear transcription factor κB; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PI 3-kinase, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLCγ, phospholipase Cγ; PtdCho-PLC, phosphatidylcholine-hydrolysing PLC; SAP, stress-activated protein; SH-2 and SH-3, Src homology-2 and -3; SHC, *src* homology/collagen; TNFα, tumour necrosis factor α; Tyk2, tyrosine kinase 2.

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Table 1 Proteins containing SH-2 and SH-3 domains within their stucture

Protein	SH-2	SH-3	Function/targets	Reference
PLCγ	Yes	Yes	PtdIns(4,5)P_2 hydrolysis	[18]
GAP	Yes	Yes	Ras GTPase	[19]
Src (*c-src, lyn, fyn, yes, lck, fgr, hck, blk, yrk*)	Yes	Yes	Tyrosine kinase/signalling in haematopoietic cells	[9]
SHPTP-1	Yes	–	Tyrosine phosphatase	[20]
SHPTP-2	Yes	–	Tyrosine phosphatase/adaptor to Grb2	[21]
VAV	Yes	Yes	Nucleotide exchange factor in haematopoietic cells	[22]
ZAP-70	Yes	–	Tyrosine kinase (T cells)	[60]
p72^{syk}	Yes	–	Tyrosine kinase (B cells)	[62]
Grb2	Yes	Yes	Adaptor to mSos	[6]
p85 PI 3-kinase	Yes	Yes	110 kDa PI 3-kinase	[23]
Nck	Yes	Yes	Unknown	[24]
SHC	Yes	–	Adaptor to Grb2	[43]
Tensin	Yes	–	Cytoskeletal protein	[25]
p47 and p67^{phox}	–	Yes	Cytochrome β_{558}	[26]
α-Spectrin	–	Yes	Cytoskeletal protein	[27]
STAT 91	Yes	Yes	ISGF complex/GAF	[28]

Table 1). Autophosphorylation is limited to a number of defined residues within specific regions of the cytoplasmic domain which determine the interaction of the receptor with each individual target protein. Initial studies revealed that partial deletion of the kinase insert domain prevented the interaction of the autophosphorylated PDGF-β receptor with PI 3-kinase but did not prevent interaction with PLCγ [2,11]. Using site-directed mutagenesis, it was shown that PI 3-kinase binds to tyrosines 708 and 719 of the mouse and tyrosines 740 and 751 of the human PDGF receptor [12,13]. Other signalling molecules such as GAP and PLCγ do not bind to these residues but interact with other distinct tyrosine residues at different sites within the cytoplasmic domain (Figure 1).

Experiments using synthetic phosphopeptides derived from amino acid sequences within the PDGF receptor have also provided additional information regarding the structural requirements for receptor–effector interactions [13–15]. The three amino acids C-terminal to the phosphorylated tyrosine residue (pTyr), play an important role in the selectivity of interaction. Fantl et al. [13] and others showed that the peptides ^{p}Y-M-D-M-S and ^{p}Y-V-P-M-L, corresponding to sequences containing tyrosines 708 and 719 respectively, specifically inhibited the binding of the murine PDGF receptor to PI 3-kinase *in vitro*, while ^{p}Y-M-A-P-Y-D-N-Y was effective in preventing the interaction with GAP [13–15]. Further studies utilizing a phosphopeptide library have now identified a large number of motifs which determine the interaction of the growth factor with each individual signalling molecule. For example Src tyrosine kinases preferentially bind peptides with the sequence pTyr-hydrophilic-hydrophilic-Ile/Pro whereas other proteins such as p85, PLCγ and SHPTP-2 each select different and unique sequences within the general motif, pTyr-hydrophobic-Xaa-hydrophobic [15]. For example, p85 selectively binds to regions with methionine in positions +1 and +3 relative to Tyr^{740} within the human PDGF receptor.

SH-2 and SH-3 domains

High-affinity interactions between growth factor receptors and effectors are also defined by specific regions within the target molecules consisting of approximately 100 amino acids. These regions are defined as Src homology-2 (SH-2) domains by virtue of their considerable sequence similarity to the non-catalytic region of the *src* family of protein tyrosine kinases [16]. The p47$^{gag\text{-}crk}$ oncoprotein was originally demonstrated to interact with a number of phosphotyrosine-containing proteins through a region containing an SH-2 domain [17]. Since that initial observation a large number of signalling molecules, including those listed above, have been shown to contain SH-2 domains which bind directly to tyrosine-phosphorylated receptors [18–28] (Table 1).

The structural features which determine the specificity of binding of SH-2 domains to tyrosine-phosphorylated growth factor receptors have recently been identified. X-ray crystallography has indicated that Arg^{175}, buried within a specific binding pocket, is critical for interaction with the phosphate group of the tyrosine residue [29]. This amino acid is conserved in all SH-2 domains and deletion or substitution of the residue results in a loss of peptide binding. Other residues around this region involved in the interaction with the phosphate group can vary. For example, Arg^{155} is replaced by Lys and Gly in GAP and SHPTP-1 respectively. An additional pocket, able to accommodate the pTyr +3 residue contained within receptor phosphopeptide sequences, is also present [30] and is likely to be required for high-affinity binding. Together the pTyr and pTyr +3 residues form a double-branched segment which can 'plug into' the SH-2 domain. A number of proteins such as p85 and PLCγ1 also contain two SH-2 domains and can potentially bind to two distinct phosphopeptide sequences within the receptor. Thus several points of interaction between the receptor and signalling molecules containing SH-2 domains can occur.

In some instances, receptor binding results in the tyrosine phosphorylation and a change in the activity of the target molecule. However, the target molecule is phosphorylated on residues distinct from those in the SH-2 domain responsible for binding to the receptor. Binding of PLCγ to the PDGF receptor on Tyr^{1021} stimulates phosphorylation upon tyrosine residues at positions 771, 783 and 1254, increasing the catalytic activity of PLCγ [31]. Similarly, tyrosine phosphorylation increases the phosphatase activity of SHPTP-2 [32] and may also enhance the activity of other factors such as GAP. However, a number of proteins such as Grb2, Nck and p85 do not possess a catalytic domain. Thus phosphorylation, if it occurs, does not result in changes in activity. These proteins merely serve as adaptor molecules to couple the activated receptor to other intermediates. Such proteins contain additional Src homology-3 (SH-3) domains composed of approximately 60 amino acid residues

containing a proline-rich sequence of 10 amino acids [33]. Both secondary and tertiary structures vary between SH-3 domains of different proteins, suggesting specificity of interaction with downstream target molecules. Studies using both biochemical and molecular approaches have provided evidence to suggest that a functional SH-3 domain is essential for GAP-mediated Ras signalling [34] and Grb2 binding to the Ras guanine nucleotide exchange factor, mammalian Son of sevenless (mSos), which links growth factor receptors to the Ras/mitogen-activated protein (MAP) kinase cascade (see below) [35]. However, binding of SH-2 domain-containing adaptor molecules to other intermediates may not always require SH-3 domain interactions, e.g. the binding of p85 to the 110 kDa catalytic subunit of PI 3-kinase (p110) is through a site contained within the inter-SH-2-domain region of p85 [36].

Recently it has been shown that SH-3 domains within proteins may also be involved in other aspects of function, particularly the targeting of molecules to specific subcellular locations. Deletion of either SH-3 domain of Grb2 prevents association of Grb2 with the plasma membrane, possibly preventing its interaction with the activated receptor, while the SH-3 domain of PLCγ is essential for its association with the actin cytoskeleton where it acts to catalyse PtdIns(4,5)P_2 hydrolysis [37]. Indeed, a number of cytoskeletal proteins, for example α-spectrin, contain SH-3 domains [27], consistent with the idea that the SH-3 domain is involved in membrane localization. SH-3 domains have also been implicated in Src and VAV interactions with RNA-binding proteins during mitosis [38], and in the binding of the p47 and p67^{phox} proteins to cytochrome β_{558} during the formation of the respiratory burst complex in neutrophils [39] (Table 1).

Insulin receptor substrate, *src* homology/collagen (SHC) and multiple signalling complexes

An important variation in the general model of growth factor–effector interactions involves the activation of the insulin signalling cascade. Although insulin receptor dimerization and autophosphorylation upon specific tyrosine residues is observed, there appears to be only weak association between the receptor and SH-2 domain-containing proteins. White and co-workers isolated and cloned a 185 kDa protein, insulin receptor substrate 1 (IRS-1) [40]. Insulin stimulates the phosphorylation of IRS-1 upon tyrosine residues within consensus sequences found to be similar to those observed for the PDGF receptor [41]. Phosphorylated IRS-1 interacts with PI 3-kinase, SHPTP-2 and Grb2 [41,42] and functions as a core signalling molecule for the insulin receptor.

In some instances, other SH-2 domain-containing proteins may serve as adaptors to provide alternative links between activated growth factor receptors and other SH-2 domain-containing effectors. Recently SHC, a ubiquitous protein that contains a single SH-2 domain and a glycine/proline-rich region but no catalytic domain, has been isolated [43]. SHC is tyrosine phosphorylated by the activated EGF receptor [44] and IRS-1 [42] and forms a stable complex with the SH-2 domains of other proteins such as Grb2 [44]. SHC may therefore act as a universal tyrosine kinase substrate adaptor protein. This may be of particular importance for pp60$^{v\text{-}src}$, middle T-antigen and for T- and B-lymphocyte antigen receptors which do not have cytoplasmic tyrosine kinase domains and cannot interact with Grb2 and other signalling molecules directly [45–47].

Recently, it has been shown that there are hierarchical binding sites for Grb2 and SHC within the EGF receptor [48]. This suggests that certain receptor–protein interactions may take place preferentially, determined by the affinity of each protein for the receptor phosphopeptide sequence and the relative cellular expression of each signalling molecule. Evidence in support of this idea is accumulating as it has now been shown that different signalling molecules, for example p85 and Nck (and possibly SHPTP-2 and PLCγ), may compete for the same phosphotyrosine residue within a single receptor [4,8,49]. Furthermore, recent evidence suggests that multiple receptor–protein interactions may be required to initiate a signalling pathway. Following binding to the activated growth factor receptor, SHPTP-2 is in turn able to bind Grb2 through a pTyr-Xaa-Asp-Xaa motif present within SHPTP-2 [50]. This occurs despite the presence of another Grb2-binding site within the kinase insert domain of the receptor [7].

Overall, these results indicate the potential for the involvement of multiple protein–protein interactions in the coupling of a single growth factor receptor to several signalling pathways. However, it remains to be determined whether these multiple receptor coupling events are observed due largely to the high levels of expression of foreign receptors in different cell types. This combined with various levels of signalling components in the target cell may result in a compromise in the law of mass action upon which the receptor–effector interaction is presumably based. For example, the interaction of the insulin receptor with SHC is only observed in cells expressing high levels of the insulin receptor [44], while in endothelial cells transfected with PDGF receptor mutants, the loss of Grb2 binding does not abrogate PDGF-induced activation of MAP kinase [7]. It is therefore likely that the coupling of a growth factor receptor to a specific signalling cascade will be far more stringently controlled *in vivo* than indicated by these studies. However, it is possible that loss of fidelity in the activation of signalling pathways may be a feature in disease states where receptors and other signalling components are overexpressed. Furthermore, these results may also indicate a degree of 'signalling pathway reserve' which is utilized when the normal pathway is compromised or which is initiated at high levels of receptor occupancy. Thus the abrogation of a single signalling protein may have little effect on the overall function of the system. This may explain why in transgenic mice lacking IRS-1, insulin-responsive cellular systems are not compromised [51].

Activation of c-Src

Although c-Src (pp60^{src}) binds to activated growth factor receptors and an increase in kinase activity is observed, it is now clear that additional mechanisms are involved in the activation of this family of protein tyrosine kinases (see Figure 2). An early indication of the potential complexity of Src regulation came from the observation that c-Src is activated by phosphatases present in cell lysates [52]. An inhibitory phosphorylation site was identified as Tyr^{527} in the non-catalytic C-terminal tail and is conserved in the majority of the Src family members. In addition, a specific C-terminal Src kinase (Csk) has been isolated [53] which phosphorylates c-Src on Tyr^{527}, resulting in an inhibition of activity. Deletion of the *Csk* gene in mice results in reduced cellular phosphorylation of c-Src within the C-terminus but enhanced kinase activity [54]. An additional tyrosine phosphorylation site (Tyr^{416}) is also present within the catalytic domain of c-Src [55] but while C-terminal phosphorylation is inhibitory, autophosphorylation within this domain stimulates kinase activity. Thus a number of activity states of Src can be achieved dependent on the relative degree of phosphorylation at these residues.

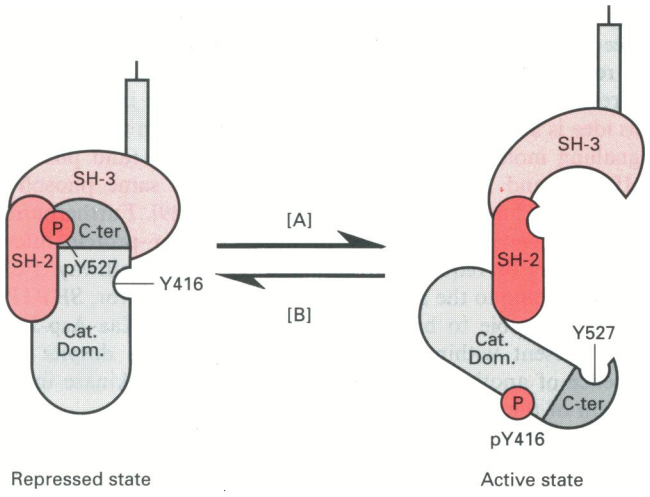

Figure 2 Schematic diagram representing the regulation of Src conformation and activation

[A], Stimulation of Src catalytic activity following autophosphorylation of Y416 and dephosphorylation of pY527 with the resulting conformational change from the repressed to the active state. [B], Inhibition of Src catalytic activity following phosphorylation of Y527 by Csk and dephosphorylation of pY416 with the resulting change from the active to the repressed state. (See refs. [52–58].) Cat. Dom., Catalytic domain; C-ter, C-terminal non-catalytic domain.

The mechanism of Src activation following growth factor receptor stimulation is still unclear. In the inactivated state, the C-terminal-phosphorylated tyrosine binds to the Src SH-2 domain. This has been suggested by studies which show that both Src or the SH-2 domain of Src expressed as a fusion protein can bind to a synthetic phosphorylated peptide modelled on the C-terminus of the Src tail [56]. X-ray crystallography has also suggested that the Src family member p56^{lck} may exist as a dimer and that the C-terminal phosphotyrosine is bound to the SH-2 domain within an opposing molecule [57]. It has been postulated that following receptor autophosphorylation, the receptor phosphopeptide sequence has higher affinity for the SH-2 domain of Src and thus is able to displace the C-terminal inhibitory phosphotyrosine. This event is thought to initiate an allosteric change in the molecule and catalyse autophosphorylation of Src upon Tyr^{416}. However, evidence for this hypothesis has not been forthcoming and since phosphorylation is essential for C-terminal–SH-2 interactions, it is likely that a receptor-associated protein tyrosine phosphatase is also involved [58]. It is also likely that other kinases and phosphatases may regulate the activity status of Src, and the finding that in the absence of Csk activity Tyr^{527} is still partially phosphorylated supports the hypothesis that an additional inhibitory kinase is present [54].

Signalling by Src family members

Although c-Src may play a role in regulating RNA trafficking or translation by binding to the GAP-associated protein p68 during mitosis [38], other members of this kinase family are also involved in the immediate activation of tyrosine kinase signalling pathways. This is particularly significant in cells of haematopoietic origin following activation of receptors which do not contain intrinsic tyrosine kinase activity. In B and T lymphocytes, the Src kinases p59^{fyn} and p56^{lck} stimulate the activation of PI 3-kinase through binding of the Src SH-3 domain to proline-rich regions within p85 [59]. In T lymphocytes, current evidence suggests that p56^{lck} can stimulate the phosphorylation of tyrosine residues within antigen recognition activation motifs (ARAMs) of the T-cell receptor complex, which promotes the recruitment of SH-2 domain-containing signalling molecules such as ζ chain-associated protein (ZAP-70) and SHC to the receptor [47,60,61] (see Table 1). These molecules may link T-cell receptor activation to downstream signalling pathways such as PLCγ1 and the p21^{ras}/MAP kinase cascade (see below). In B cells a homologous protein, known as spleen tyrosine kinase (p72^{syk}), plays a similar role, coupling p59^{fyn} activation to a number of downstream signalling events [62].

It remains to be determined whether c-Src can also facilitate the activation of molecules such as p85 and SHC in response to either growth factor or G-protein-coupled receptor stimulation. Activation of both types of receptor can result in the activation of both Src kinase [10,63] and PI 3-kinase activity (see below), although the two responses may not necessarily be linked. In fibroblasts, however, the SH-3 domain of Src can bind to SHC, suggesting the possibility of this interaction at least for growth factor receptors [64].

GROWTH FACTOR-INDUCED ACTIVATION OF THE MAP KINASE CASCADE

Concomitant to the investigations into the interactions between growth factor receptors and SH-2/SH-3-domain-containing proteins has been the elucidation of the pathways downstream of these initial signalling events. One such cascade characterized in this way is the MAP kinase pathway. MAP kinases or extracellular regulated kinases (ERKs) has been proposed to play an important role in cell growth and differentiation stimulated by growth factors, G-protein-coupled receptor agonists, cytokines and antigens. Following activation, MAP kinase translocates to the nucleus [65] and initiates the activation of nuclear proto-oncogenes such as c-*jun* [66]. Other cellular events activated by MAP kinase include the phosphorylation of p90 ribosomal S6 kinase (p90^{S6k}), cytosolic phospholipase A_2 (cPLA$_2$), MAP kinase-activated protein (MAPKAP) kinase-2 and the stimulation of glucose transport (reviewed recently in [67]).

The elucidation of the mechanisms that regulate MAP kinase activation has advanced greatly over recent years due to the finding that the MAP kinase cascade is highly conserved within eukaryotes. The activation of a homologous set of signalling molecules has been identified through genetic analysis of the pathway that controls vulval development in the nematode worm, *Caenorhadbitis elegans* (*C. elegans*) [68], the photoreceptor R7 [69] and torso receptor protein tyrosine kinase pathways within the fruit fly *Drosophila melanogaster* [70,71], and pheromone response protein kinase pathways in yeast strains such as *Schizosaccharomyces pombe* (*S. pombe*) [72] and *Saccharomyces cerevisiae* (*S. cerevisiae*) [73,74]. These studies and additional biochemical approaches have directly led to the identification of the mammalian counterparts (Figure 3).

MAP kinase was first identified by Ray and Sturgill [75] as a serine/threonine-directed kinase that utilized microtubule-associated protein 2 as a substrate following stimulation of adipocytes with insulin. MAP kinase activity was mediated by two proteins of approximate molecular mass 42 kDa which had been observed to be phosphorylated upon tyrosine residues in response to growth factors and phorbol esters [76–78]. Cloning studies now indicate the presence of at least two isoforms of MAP kinase, i.e. pp42 and pp44, with a third, pp54, having approximately 50% sequence similarity with its counterparts [79,80].

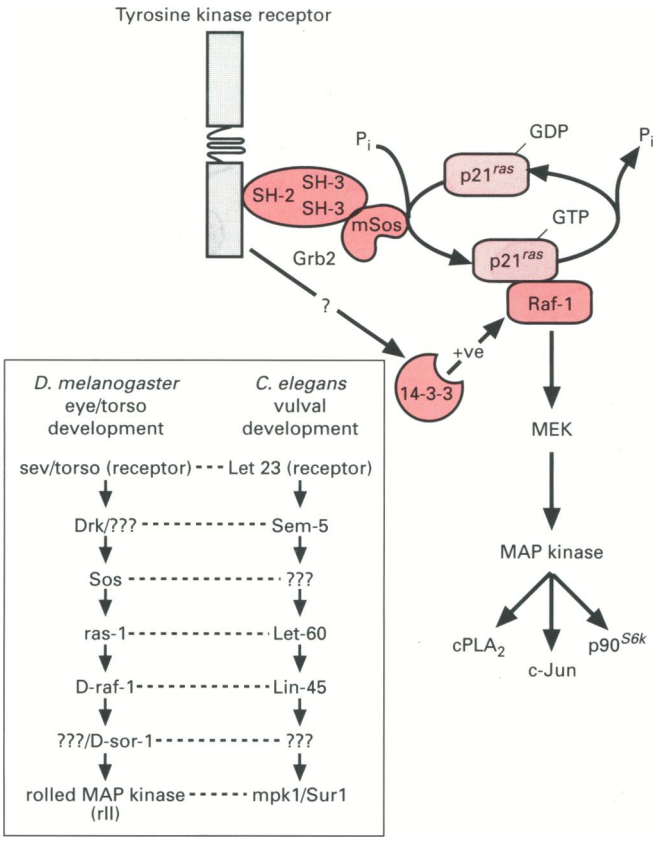

Figure 3 Growth factor signalling through the MAP kinase cascade

Inset: an example of the conserved nature of the MAP kinase cascade in eukaryotes. Broken lines represent homologous proteins. ??? represents undetermined intermediates. (See refs. [65–127].)

The activation of MAP kinase requires phosphorylation upon both tyrosine and threonine residues [81,82] by the dual specific kinase, designated as MAP kinase kinase or MEK (MAP kinase or Erk kinase) [83,84]. Phosphorylation of pp42 MAP kinase occurs upon Thr^{183} and Tyr^{185} residues in the mammalian and Thr^{188} and Tyr^{190} in *Xenopus* isoforms [81,85]. Phosphorylation of both residues is essential for enzyme activation as single point mutation (Thr^{183} → Ala; Tyr^{185} → Phe) experiments produce inactive mutants [86]. Recently the three-dimensional atomic structure of MAP kinase has been resolved in its unphosphorylated, low-activity conformation [87]. Noticeably, Thr^{183} is on the surface of the molecule and well ordered while Tyr^{185} is buried in a large hydrophobic pocket and blocks the peptide-binding site, suggesting that activation is likely to involve both global and local conformational changes. Kinetic analysis of the rate of incorporation of phosphate into Tyr and Thr residues of MAP kinase by the action of MEK suggested that the enzyme is phosphorylated first on the tyrosine residue [87]. This implies that the initial change in conformation of the enzyme occurs upon the binding of MEK to MAP kinase and not upon phosphorylation of Thr^{183}. Such domain motions are widely used in nature to regulate enzyme activity [88].

MEK is related in sequence to the yeast protein kinases from *S. pombe*, Byr1 kinase and *S. cerevisiae* STE7 kinase [89], which have been proposed to function upstream of Spk1 and KSS1/FUS3 (homologues of vertebrate MAP kinase) in the pheromone-induced signalling pathway [90]. Two main isoforms of MEK have been identified in mammalian cells, MEK1 and MEK2, as well as an alternative splice variant of MEK1, MEK1β [91,92] or MEK3 [93], which lacks an internal domain of 26 amino acids.

MEK is activated by phosphorylation [94,95] exclusively on Ser^{218} and Ser^{222} by c-*raf*-1, the cellular homologue of the v-*raf* oncogene [96–98]. Raf has for a number of years been implicated in the regulation of cell proliferation by growth factors and oncogenes. Overexpression of c-*raf* or v-*raf* in fibroblasts results in constitutive activation of MAP kinase and MEK while MEK, deactivated by phosphatase 2A, is re-activated *in vitro* by c-Raf-1 immunoprecipitated from stimulated cells. Three mammalian Raf isoforms have now been identified with associated serine/threonine kinase activity (c-Raf-1, A-raf and B-raf). c-Raf-1 is ubiquitously expressed while A-raf and B-raf have tissue-specific distributions [99].

MAP kinase has also been shown to phosphorylate MEK, but on both Ser and Thr residues [100]. The functions of these different phosphorylation events are as yet not known. However, in the case of MAP kinase they may be the result of 'feedback' signalling [101]. Recently, Rossomando et al. [102] reported on the negative regulation of MEK1 but not MEK2 enzyme activity following phosphorylation of two threonine residues, Thr^{286} and Thr^{292} (residues that are not present in MEK2), *in vitro* by p34^{cdc2}, a serine/threonine protein kinase that is involved in cell cycle regulation. MEK1 isolated from HeLa cells was also found to be phosphorylated upon these residues. These results suggest that MEK1 and MEK2 may be regulated differently and that phosphorylation events play a critical role not only in the activation but also in the negative regulation of MAP kinase activity within this cascade.

Ras–Raf interactions

A major advance achieved in characterizing the MAP kinase cascade was the identification of a role for p21^{ras}. The *ras* proto-oncogene product, a 21 kDa molecular-mass G-protein, was well recognized to play a role in cell growth and transformation. However, its position in a signalling cascade sequential or parallel to Raf had not been clarified. The discovery of a Ras-GAP by McCormick and colleagues, which negatively regulated Ras activity, implicated the dysfunction of Ras-GAP activity as a potential mechanism in cellular transformation [103,104].

Following the identification of MAP kinase, however, it was discovered that the expression of oncogenic *ras* could activate MAP kinase while a dominant negative mutant of *ras*, such as N17 or M17 *ras*, blocked activation of both Raf [105] and MAP kinase [106] in response to growth factors. In addition, growth factor or *ras* oncogene activation of MAP kinase and downstream signalling events could be abolished following expression of dominant negative c-*raf*-1 mutants or application of Raf anti-sense RNA [107–109]. These observations, coupled with knowledge of Raf and MEK interactions [110], position Ras upstream of Raf in the MAP kinase cascade.

Recent studies have implicated an additional degree of complexity in the activation of Raf. A number of groups have now shown that the interaction of Ras with Raf is direct and no intermediate steps are involved [111]. The N-terminus of c-Raf-1 expressed as a glutathione S-transferase fusion protein binds directly to Ras. Native Raf binds only to GTP-bound Ras or the constitutively active Ras mutant Gly-12Val but does not bind the effector domain mutant Ile-36Ala [112,113]. However, the binding of Ras is not sufficient to activate Raf since the requirement for Ras in the activation of Raf can be circumvented by the attachment of C-terminal membrane localization signals of Ras (CAAX sequences) to c-Raf-1, thereby targetting Raf to

the plasma membrane [114,115]. Therefore, this suggests that the function of Ras is to target Raf to the plasma membrane where it can be activated by some other mechanism. Recently, two members of a protein family designated as 14-3-3 have been found to interact with Raf and regulate its activation [116,117], although as yet it is not clear whether these proteins or phospholipid mediators (see below) provide a link between growth factor receptors and MAP kinase activation.

Grb2–mSos interactions

Other intermediates linking activated growth factor receptors to the activation of Ras have recently been identified, largely from studies of the homologous signalling system in the *Drosophila* sevenless receptor protein-tyrosine kinase pathway and *C. elegans* vulval development pathways. The Ras activator is the mammalian homologue of *Drosophila* Son of sevenless, referred to as mSos [118,119]. Sos proteins are also homologous to several yeast nucleotide exchange factors which induce guanine nucleotide exchange on yeast Ras proteins [119]. Recently the mammalian homologue has been shown to perform the same function, confirming that mSos is a guanine nucleotide release protein required for Ras signalling. Consistent with this finding are a number of previous studies which suggest that stimulation of guanine nucleotide exchange activity may be the primary mechanism of Ras activation by several growth factors, including nerve growth factor (NGF) and PDGF [120,121].

Grb2 has also been implicated in the regulation of Ras activity following the observation that microinjection of Grb2 in concert with $p21^{ras}$ induced proliferation of rat embryo fibroblasts [6]. Homologous proteins of Grb2 also exist, Drk in *Drosophila* and Sem-5 in *C. elegans*, and both are recognized to be 'upstream' regulators of Ras activation [122,123]. Clark et al. [124] showed that mutations in Sem-5 can be rescued by activated *ras* alleles, indicating that this protein functions upstream of Let-60 *ras*. It has also been demonstrated that Drk is essential for the normal development of the *Drosophila* photoreceptor and that the constitutive activation of Ras bypasses the normal requirement for Drk function [123]. Drk can also associate with both the sevenless receptor and Sos (Figure 3 inset).

Using site-directed mutagenesis and peptide competition experiments, a number of groups have shown that Grb2 binds directly to mSos via proline-rich regions in the C-terminal region of mSos [35] and that high-affinity binding requires both SH-3 domains of Grb2. A conserved arginine residue within the proline-rich binding region of mSos may confer some specificity to the interaction between mSos and Grb2, as substitution to a Lys residue in this position abolishes the ability of a peptide generated from this region to compete with mSos for binding to Grb2 [125–127]. Grb2/mSos complexes activate nucleotide exchange activity on $p21^{ras}$ *in vitro* [35,126,127] which initiates binding to Raf-1. This process, possibly in combination with interactions with 14-3-3 proteins, results in the downstream activation of Raf.

ACTIVATION OF THE MAP KINASE PATHWAY IN RESPONSE TO G-PROTEIN-COUPLED RECEPTORS

The activation of the MAP kinase cascade and other tyrosine kinase pathways (see below) in response to agonists whose receptors are coupled to G-proteins have recently been investigated, largely to clarify their status as mitogens. Although some G-protein-linked receptor agonists (G-LRAs) were found to strongly stimulate polyphosphoinositide hydrolysis and the activation of PKC, this was deemed not to be sufficient to initiate cell cycle progression [128,129].

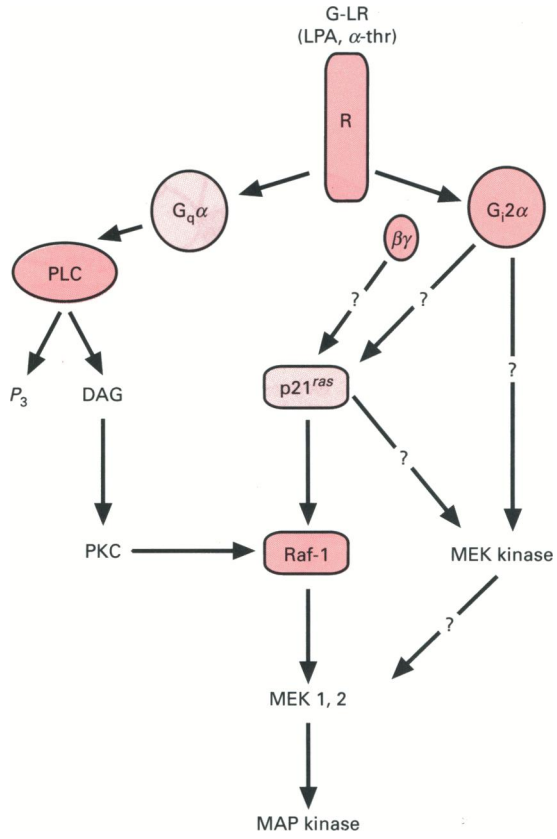

Figure 4 Schematic diagram representing pathways involved in G-protein-coupled receptor activation of the MAP kinase cascade

See refs. [128–156].

The implication of MAP kinase as being central to mitogenesis [129,130] has led to experiments to discover whether G-LRAs could stimulate MAP kinase. A number of groups have shown that G-LRAs can stimulate the activation of MAP kinase, an activation that now appears to occur in most cell types. For a large number of these agonists, PKC has been found to be intimal to the activation of MAP kinase [131,132]. Agonist-induced activation of MAP kinase is closely associated with the activation of PKC and in most instances can be inhibited by down-regulation of PKC by chronic phorbol ester pretreatment or preincubation with PKC inhibitors. Consistent with this finding is the observation that PKCα can phosphorylate Raf-1 upon serine residues *in vitro* [133] (Figure 4).

However, other agonists such as thrombin and the mitogenic phospholipid, lysophosphatidic acid (LPA), have been shown to activate the MAP kinase pathway [129,134–136] by a mechanism which is believed to be independent of changes in PKC activation. In these instances MAP kinase activation has been shown to be pertussis toxin-sensitive and this sensitivity is reflected at the level of cell division. In Rat-1 fibroblasts the tyrosine phosphorylation of MAP kinase by both thrombin and LPA is associated with the pertussis toxin-sensitive activation of nucleotide exchange on $p21^{ras}$ [134–136]. Overexpression of c-*raf*-1 potentiates LPA-stimulated MAP kinase activation while overexpression of a dominant negative mutant of $p21^{ras}$ inhibits LPA-induced Raf-1 and MAP kinase activation, demonstrating the involvement of both Ras and Raf in the LPA-activated MAP

kinase cascade [137]. The G-protein likely to be involved in the activation of $p21^{ras}$ is G_i2, since transfection of cells with the GTPase-deficient $G_i2\alpha$ subunit oncoprotein, *gip*2, results in the constitutive activation of MAP kinase [138,139]. Furthermore, in cells expressing high levels of receptor coupled to G_i2 activation, such as the α_{2A}-adrenoreceptor or the muscarinic M_2 receptor, a pertussis toxin-sensitive activation of $p21^{ras}$ and MAP kinase is observed [140,141].

Recent evidence favours a role for G-protein $\beta\gamma$ subunits in the activation of Ras by G-LRAs. In Cos-7 cells transfected with a mutant $G_i\alpha$ that is constitutively active no activation of MAP kinase occurred; however, overexpression of $\beta\gamma$ subunits, particularly $\beta1\gamma2$, was found to stimulate MAP kinase [142,143]. Previously $\beta\gamma$ subunits have been shown to mediate the pheromone receptor activation of FUS3/KSS1 in *S. cerevisiae*, although in this case the pathway is Ras-independent [144]. In mammalian cells, Crespo et al. [143] have proposed that the $\beta\gamma$ subunits may transduce the signal via a similar mechanism to the activation of the β adrenergic-receptor kinase which binds free $\beta\gamma$ heterodimers through a region that contains a pleckstrin homology (PH) domain [145]. A number of proteins known to regulate Ras activity also express a PH domain [146], thus free $\beta\gamma$ subunits may affect the function of Ras by binding the PH domain of one or more Ras-regulatory proteins. Significantly, it has also been demonstrated that membrane localization of $\beta\gamma$ subunits is an absolute requirement to induce the activation of MAP kinase. This suggests that the targeting of proteins containing PH domains to the plasma membrane by $\beta\gamma$ subunits may be a critical event in Ras activation.

It should be noted that overexpression of $\beta\gamma$ subunits or receptors linked to G_i2 is normally required to activate $p21^{ras}$ and MAP kinase, thus it is more likely that the physiological role of $\beta\gamma$ subunits may be to enhance the activation of $p21^{ras}$ following stimulation of an endogenous receptor, such as LPA, by recruitment of additional intermediates to the plasma membrane for activation by some other mechanism. The finding that LPA-induced activation of $p21^{ras}$ is inhibited by tyrosine kinase inhibitors [135,136] but that the response to muscarinic M_2 receptor stimulation is unaffected [141] also suggests that additional tyrosine kinase intermediates may be required for the activation of $p21^{ras}$ by G-protein-coupled receptor agonists.

Raf-INDEPENDENT ACTIVATION OF MAP KINASE

MEK kinase

While evidence in support of PKC-mediated phosphorylation and activation of Raf is substantial, a number of recent studies have indicated that this may not be the sole mechanism for the activation of MAP kinase. In contrast to earlier studies which used peptide pseudosubstrates to measure the activity of Raf-1 [135], MacDonald et al. [147] have shown that although Raf-1 is phosphorylated by PKC *in vitro* it is unable to phosphorylate purified inactive MEK. Other studies have also indicated that the activation of the Ras/Raf pathway may not be the only pathway utilized by different growth factors to activate MAP kinase [148]. An alternative pathway involved in the regulation of MAP kinase by both growth factors and G-protein-coupled receptors has now been implicated [149,150] (Figure 4). Stimulation of the heterotrimeric G-protein-linked thrombin receptor or expression of the $G_i2\alpha$ mutant, *gip*2, both activate MEK in a Raf-independent manner in Rat-1 fibroblasts [138]. Johnson and co-workers isolated a serine/threonine kinase of 74 kDa which phosphorylates MEK *in vitro*, originally identified by virtue of its homology to two known MEK kinases STE11 and Byr2 isolated from *S. cerevisiae* and *S. pombe* respectively [149,150]. c-Raf-1 is unrelated to STE11 and Byr2 and therefore may represent, in mammalian cells, a divergence from the pheromone response protein kinase system defined in yeast. Phosphorylation events within MEK induced by MEK kinase and Raf-1 are not identical [93,94]. MEK kinase preferentially phosphorylates Ser^{218} while Raf-1 shows no preference for either Ser^{218} or Ser^{222}. These different Raf- and MEK-kinase-mediated events may represent separate mechanisms of MEK activation [151].

To date no studies have demonstrated unequivocally, agonist activation of MEK kinase by standard immunoprecipitation and *in vitro* kinase techniques. The implication of MEK kinase involvement in the regulation of MAP kinase is at the moment circumstantial. Nevertheless it has been shown in NRK cells that expression of c-*raf*-1 antisense does not affect MAP kinase activation by both PDGF and EGF [152]. In addition, it has been found that in both Swiss 3T3 cells and PC12 cells there are at least two different growth factor-sensitive MEK activators, one recovered from anti-Raf-1 immunoprecipitates and a second activity that is distinct from Raf [153]. This Raf-independent MEK activator appears to play a major role in the activation of MEK as c-Raf-1 constitutes only 20% and 5% of the total MEK-activating activity in EGF-treated Swiss 3T3 cells and NGF-treated PC-12 cells respectively. A recent study has implicated MEK kinase downstream of $p21^{ras}$ in NGF-stimulated PC-12 cells [154], although this remains to be determined for other cell types.

At present the physiological regulation of MEK kinase is poorly understood and no consensus has been reached as to its precise role in the MAP kinase and related cascades. In yeast (*S. cerevisiae*), expression of the catalytic domain of MEK kinase was able to overcome a PKC deletion mutation [155] while 'full-length' MEK kinase failed to suppress the same mutation. From this it has been proposed that the MEK kinase N-terminal domain, which contains several consensus PKC phosphorylation sites, may function as a negative regulatory domain and that PKC phosphorylation may provide one mechanism for activating MEK kinase, and indeed these findings are consistent with a role for PKC in a Raf-independent MAP kinase pathway. However, these findings must be treated with caution since Yan et al. [156] have now demonstrated that overexpression of MEK kinase in NIH 3T3 cells results in the specific activation of an additional kinase, stress-activated protein/ERK kinase-1 (SEK-1), believed to form part of an alternative stress-activated protein (SAP) kinase cascade (see below).

REGULATION OF MAP KINASE ACTIVATION BY PHOSPHOLIPID AND CYCLIC NUCLEOTIDE SIGNALLING SYSTEMS

Phospholipid pathways

While it is accepted that the classical PKC isoforms play a role in the activation of MAP kinases, it has recently been suggested that a number of phospholipid molecules may directly regulate the MAP kinase pathway and contribute to the assembly of its components. As described earlier, activation of Raf is not achieved solely by physical association with activated Ras, but involves other Ras-independent events. This may rely upon the cysteine-rich, lipid-binding domain of Raf allowing specific lipid activation. Moscat and co-workers have implicated a phosphatidylcholine-hydrolysing PLC (PtdCho-PLC) activity to be involved in Ras and Raf coupling [157]. Transfection of *Bacillus cereus* PtdCho-PLC into NIH 3T3 cells stimulates cell growth and bypasses the expression of dominant negative *ras* mutations, yet fails to bypass the co-expression of dominant negative c-*raf* mutations [157]. This may represent the direct

activation of Raf by phosphatidylcholine-derived DAG or the activation of an unidentified Raf kinase. It is unclear whether these findings extrapolate to agonist activation of MAP kinase in normal cells since evidence in support of agonist-stimulated PtdCho-PLC catalytic activity is still circumstantial. In addition, there is a clear disparity for both growth factor and G-protein-coupled receptors, between the magnitude and kinetics of DAG formation derived from this source and the stimulation of MAP kinase.

Recently another phospholipid, PtdIns(3,4,5)P_3, the immediate product of PI 3-kinase (see below), has been proposed to play a similar role in insulin-stimulated L6 skeletal muscle cells [158] based on the finding that wortmannin, a potent PI 3-kinase inhibitor, was able to block MAP kinase activation. However, it is unclear if this will contribute as a universal mechanism of activation as synthetic PtdIns(3,4,5)P_3 failed to activate c-Raf expressed in Sf9 insect cells [158]. The recently discovered 14-3-3 proteins interact with the cysteine-rich lipid-binding region of Raf immediately C-terminal to the Ras-binding domain [116] and it is possible that these phospholipids, or related products such as arachidonate, or other proteins, may facilitate the binding of 14-3-3 to Raf. To date no *in vitro* studies utilizing 14-3-3 proteins in combination with phospholipid molecules have confirmed this hypothesis.

Ceramide-activated and stress-activated protein kinases

An alternative lipid signalling pathway which may be involved in the activation of MAP kinase is the sphingomyelinase/ceramide pathway. This cascade is activated in response to a number of cytokines, particularly tumour necrosis factor α (TNFα) and interleukin (IL)-1 (reviewed in [159]), and is associated with the activation of both the nuclear transcription factor κB (NFκB) and a number of proline-directed serine/threonine kinases. This is likely to include MAP kinase since TNFα has been shown to activate MAP kinase as well as NFκB in myeloid HL-60 cells [160,161]. Both Ras and Raf have also been implicated in the regulation of NFκB since expression of a dominant negative allelle of c-*raf*-1 inhibits TNFα-induced activation of NFκB [162]. However, the activation of MAP kinase is not thought to be upstream of NFκB, the pathway potentially bifurcating at the level of Raf. The activation of both NFκB and MAP kinase in response to TNFα is clearly Ras-independent, implicating the involvement of a ceramide-sensitive kinase in the direct activation of Raf by these agents.

The activation of a ceramide-dependent kinase pathway may also involve intermediates of the recently discovered SAP kinase cascades. This includes additional members of the MAP kinase family p38 [163–165] and the p54 c-*jun* N-terminal kinases, which have limited homology to pp42 and pp44 MAP kinase [166]. Indeed, these kinases have been shown to be strongly activated not only by cellular stress but also by lipopolysaccharide, heat shock, IL-1 and TNFα; agonists known to utilize the ceramide-activated signalling cascades. The SAP kinase cascade is stimulated poorly in response to classical mitogens such as NGF, EGF and fibroblast growth factor (FGF), indicating some degree of conservation between the cascades as has been indicated by studies in eukaryotes. However, *in vitro* experiments have indicated that components of the MAP and SAP kinase pathways may interact and it is likely that a degree of pathway 'cross-talk' may be manifest under certain conditions of supramaximal stimulation. It has recently been shown that although over-expression of MEK kinase results in the specific activation of SAP kinase and the immediate upstream activator SEK-1 [156], MEK kinase itself is still functionally downstream of Ras. This suggests that the cross-talk may involve co-regulation of Ras itself. It is possible that under normal conditions Ras regulates Raf-1 function, but following stress activation directs the cascade down the MEK kinase/SAP kinase pathway. This may be manifest as different functional responses by the cell.

Cyclic AMP regulation of MAP kinase activity

Recent studies assessing agonist activation of MAP kinase have provided a biochemical rationale for an opposing role of cyclic AMP in cell growth and differentiation in specific cell types. A number of groups have now demonstrated that in Rat-1 and NIH 3T3 fibroblasts, raising intracellular cyclic AMP abolishes activation of MAP kinase in response to growth factors [167,168]. The response to both PKC-activating phorbol esters and LPA is also inhibited in a similar manner and implicates a site of regulation common to all agents which activate MAP kinase. Consistent with these observations is the finding that inhibition of MAP kinase activity coincides with the PKA-mediated phosphorylation of c-Raf-1 within the regulatory domain [169], which results in reduced binding of c-Raf-1 to p21^{ras} [170]. Other PKA-dependent mechanisms may also be involved in the regulation of the MAP kinase cascade; for example, phosphorylation and activation of p21^{rap}, a low-molecular-mass G-protein which may compete with p21^{ras} for Raf [134], may also result in reduced MAP kinase activation. However, irrespective of the precise site and mechanism of the inhibition these findings are consistent with studies which show reversion of *ras*-induced transformation by activated $G_s\alpha$ [171].

Although PKA-mediated inhibition of MAP kinase activity has been shown in certain fibroblast cells, and in several other cell types [172], the phenomenon is not universal. In Swiss 3T3 fibroblasts [167] and in the EAhy 926 endothelial cell line [173], no acute inhibition of growth factor-stimulated MAP kinase is observed. In neuronal cells, such as PC-12 cells, agents which directly raise cyclic AMP or agonists which stimulate adenylate cyclase activate MAP kinase and induce differentiation [174,175]. This suggests either the presence of multiple Raf isoforms, some of which are positively regulated by PKA, or the presence of Raf-independent mechanisms involved in the regulation of MAP kinase as implicated above. A recent study in PC-12 cells has shown that raising cyclic AMP abolishes growth factor-activated c-Raf-1 and B-raf activity but does not affect MEK and MAP kinase activation [176]. While this study is difficult to interpret in the light of other studies which show an activation of MAP kinase under these conditions, it nevertheless provides additional circumstantial evidence in support of a Raf-independent MEK kinase which is not adversely affected by raising intracellular cyclic AMP.

Other mechanisms may also be involved in the inhibition of the MAP kinase cascade, in particular induction of the MAP kinase phosphatases, 3CH134 and PAC 1 [177,178], and it is certainly possible that these and other MAP kinase phosphatases are also induced by activation of the cyclic AMP/PKA pathway. If this is the case then the effect of cyclic AMP upon the MAP kinase cascade may be dependent on the relative influence of Raf and MAP kinase phosphatase on the activity status of MAP kinase at any point in time following agonist stimulation. Thus cyclic AMP may affect prolonged activation of MAP kinase but not short-term activation [173].

FOCAL ADHESION KINASE (FAK) pp125^{FAK}

It is now accepted that changes in cytoskeletal structure are crucial to a number of cellular events associated with growth and

division. The initiation of growth *in vivo* is often dependent upon interactions with extracellular matrix or other cells and changes in cell shape and motility. These changes are associated with the modulation of phosphorylation status of a number of enzymes or proteins associated with the cytoskeleton such as the **m**yristoylated **a**lanine-rich **C**-**k**inase **s**ubstrate (MARCKS) proteins, actin, myosin, profilin and others (see [179] for review).

A number of early studies assessing agonist-stimulated tyrosine phosphorylation in cells indicated the presence of several highly abundant proteins [180–183]. One of these proteins corresponds to the 125 kDa FAK [184], originally isolated by Parsons and co-workers in v-*src*-transformed chicken embryo fibroblasts [185]. Following agonist stimulation, FAK is found at cellular focal adhesions, co-localized with a number of other cytoskeletal proteins such as talin and paxillin [186]. Phosphorylation of these proteins is kinetically downstream of FAK activation [187,188], suggesting that this tyrosine kinase plays a role in regulating cytoskeletal assembly. This may be important in the initiation of mitogenesis since in v-*src*-transformed cells hyper-phosphorylation of FAK is observed and under these conditions cell growth no longer requires adherence to an extracellular matrix [189].

Cloning studies on FAK reveal little homology with other tyrosine kinases, no acylation sites and, significantly, an absence of both SH-2 and SH-3 domains [184]. Growth factor-stimulated tyrosine phosphorylation of FAK therefore requires the activation of additional intermediates. An involvement of c-Src has been implicated previously on the basis that overexpression of v-*src* markedly increases the tyrosine phosphorylation of FAK in fibroblasts. A number of putative Src phosphorylation sites within FAK have been identified, namely, tyrosine residues at positions 407, 576 and 577 and phosphorylation of these residues is required for full kinase activity [190]. However, it has also been shown that FAK binds to Src within its SH-2 domain only following initial autophosphorylation on Tyr^{397}[191]. Thus an initial, as yet unidentified, upstream event appears to be required for FAK autophosphorylation before full activation by Src can occur. This suggests that either the activation of Src and FAK are independent events or that FAK may facilitate the initial activation of Src by displacement of the C-terminal phosphotyrosine from the SH-2 domain of Src. In addition it has been shown that FAK binds the Src regulatory kinase Csk [192] and thus FAK may also play a role in presenting Src to Csk for inactivation. Consistent with this hypothesis is a recent study which shows that the activity of FAK and c-Src negatively correlate during different phases of the cell cycle [193].

A number of G-protein-coupled receptor agonists such as bombesin and endothelin-1, which stimulate a strong activation of polyphosphoinositide hydrolysis, also stimulate a marked increase in the tyrosine phosphorylation of FAK in fibroblast cell lines. However, although FAK contains a canonical PKC sequence, several studies indicate that agonist activation of FAK is at least partially, and in some instances completely, PKC-independent [182,183,194]. An increase in the tyrosine phosphorylation of FAK is also stimulated by LPA in fibroblasts and in other cell lines, but the response is not affected by pertussis toxin pretreatment at concentrations where MAP kinase activation is reduced, suggesting no involvement of a $G_i\alpha$-activated pathway [183]. However, this finding may be cell-type-specific since the tyrosine phosphorylation of FAK in neonatal fibroblasts is inhibited by pertussis toxin pretreatment and G-protein γ subunits have been located at focal adhesions by immunofluorescence [195,196].

In addition to mediating changes in cytoskeletal structure, low-molecular-mass G-proteins of the *rho* subclass have been implicated in the regulation of FAK tyrosine phosphorylation. Inactivation of *rho* by C3 exoenzyme-catalysed ADP ribosylation results in a reduction of LPA-stimulated tyrosine phosphorylation of FAK [197]. Previously, *rho* has been shown to stimulate the formation of stress fibres and focal adhesions [198] but it is at present unclear whether *rho* directly interacts with FAK or initiates changes in the cytoskeleton and so alters the phosphorylation status of FAK indirectly. Furthermore, disruption of the cytoskeleton by pretreatment of cells with cytochalasin D abolishes the tyrosine phosphorylation of FAK in response to phorbol ester or agonist activation. This suggests that either the targeting of FAK to appropriate sites within the cell is necessary for activation, or that disruption of the cytoskeleton affects the intermediate tyrosine kinase which is required for FAK activation. This may also depend on serine-directed phosphorylation of the non-catalytic C-terminus of FAK [199], the region which is responsible for subcellular localization, and may also explain why PKC-mediated tyrosine phosphorylation of FAK is a feature in some cell types.

In addition to agonist stimulation, tyrosine phosphorylation of FAK is also observed in response to integrin activation following addition of anti-integrin antibodies or by adherence to components of the extracellular matrix [200]. In platelets, tyrosine phosphorylation of FAK normally requires aggregation and involves activation of the integrin GpIIb–IIIa by fibrinogen binding to adjacent platelets [201,202]. Activation and translocation of c-Src to the cytoskleleton is also stimulated by integrins in platelets [203], although again no clear interaction between Src and FAK has been established. In thrombasthenic platelets, lacking the GpIIb–IIIa complex, tyrosine phosphorylation of FAK in response to thrombin is abolished [201] but, in contrast, the tyrosine phosphorylation and activation of c-Src in response to thrombin is increased [204]. However, this activity is no longer associated with the cytoskeleton, which supports the hypothesis that one of the functions of FAK may be to target c-Src to specific membrane locations in order to terminate its activity for both growth factor and G-protein-coupled receptors.

While it is unlikely that FAK plays a role in the regulation of MAP kinase in response to either G-protein-coupled receptor agonists or growth factors, a recent study has shown that following integrin clustering, FAK interacts with Grb2 through a SH-2 consensus binding site at $pTyr^{925}$ and provides a link between the integrin receptor and the Ras/MAP kinase cascade [205]. Other molecules which interact with FAK include p85 and PLCγ, although it is unclear whether these molecules bind to $pTyr^{925}$ or some other phosphopeptide motif within the C-terminus of FAK. Phosphorylation of FAK upon Tyr^{925} is mediated by Src kinase activity, again indicating the pivotal role of these kinases in the regulation of FAK activity and subsequent signalling events.

PI 3-KINASE

Although the binding of PI 3-kinase to activated growth factor receptors has been well characterized (see above), recent advances have been made in understanding the regulation of PI 3-kinase activation and the potential role of its product, PtdIns(3,4,5)P_3, in downstream signalling events (Figure 5). Growth factor-stimulated formation of 3′-phosphorylated phosphatidylinositides, including PtdIns(3,4,5)P_3, was originally demonstrated by Cantley and co-workers [206,207]. Initially, the enzyme activity associated with this response could not be demonstrated to discriminate between different 3′-labelled phosphoinositides as potential substrates. However, a series of acute labelling studies by Stephens and colleagues [208,209] indicated that

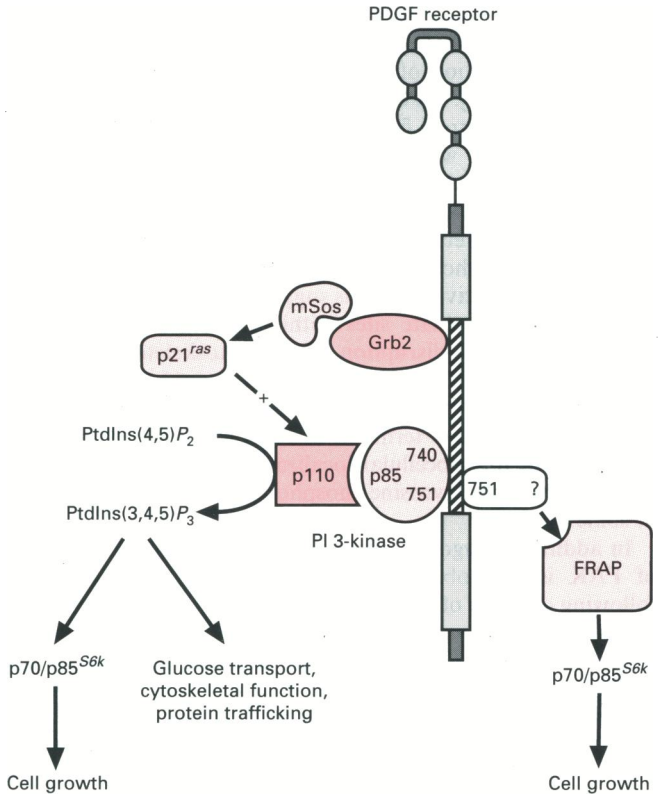

Figure 5 Regulation of growth factor receptor activation of PI 3-kinase and FRAP (rapamycin-associated protein)

See refs. [206–222].

following agonist stimulation, the formation of PtdIns(3,4,5)P_3 was achieved by direct phosphorylation of PtdIns(4,5)P_2 rather than by interconversion of other phospholipids. This confirmed the presence of an agonist-regulated PtdIns(4,5)P_2 kinase in intact cells.

PI 3-kinase has been proposed to mediate a number of intracellular events including the PKC-independent serine phosphorylation and activation of a ribosomal S6 kinase (rsk) family designated p70/85^{S6k}, which is believed to be required for the transition of the cell from G1 into S-phase [210]. A number of growth factors such as PDGF and insulin stimulate the sustained activation of p70^{S6k} and this response is inhibited by the PI 3-kinase inhibitors, wortmannin and LY294002 [211,212].

PI 3-kinase is also involved in the regulation of protein trafficking and aspects of cytoskeletal function. Injection of phosphopeptides designed to interact with the SH-2 domain of p85 results in the acute stimulation of glucose transport in *Xenopus* oocytes [213], while other studies have established a role for PI 3-kinase in the translocation of GLUT 4 glucose transporters to the plasma membrane in insulin-stimulated adipocytes [214]. Other events associated with PI 3-kinase activation include platelet aggregation [215], endothelial cell membrane ruffling [216], the release of histamine from RBL-2H3 rat basophilic leukaemia cells [217] and, intriguingly, the internalization of the PDGF receptor itself [218].

A number of studies have now implicated the potential for multiple mechanisms to be involved in the coupling of growth factor receptors to PI 3-kinase and downstream signalling events. Mutation of the PDGF receptor at Tyr^{740} abolishes receptor-associated PI 3-kinase activity [219]. However, the activation of p70^{S6k} under this condition is not affected. This suggests that the binding of p85/p110 PI 3-kinase to the PDGF receptor is not necessarily required for the activation of p70^{S6k}. In addition, mutation at Tyr$^{740/751}$ or Tyr^{751} alone does result in the abolition of p70^{S6k} activity, suggesting that another unknown intermediate protein, possibly Nck which also binds to Tyr^{751} of the PDGF receptor [49], mediates the activation of p70^{S6k}.

Despite these findings, evidence still suggests that PI 3-kinase is involved in the regulation of p70^{S6k}. Insulin-mediated stimulation of glucose transport and p70/p85^{S6k} can be dissociated by the macrolide rapamycin [220]. The intracellular target for rapamycin, following binding to its intracellular receptor FKBP12, is believed to be a rapamycin-associated protein (FRAP) [221]. FRAP is known to have limited sequence identity with the yeast gene products TOR1 and TOR2 and mammalian PI 3-kinase. Although FRAP has not yet been shown to have PI 3-kinase activity, inhibition of FRAP activity correlates with the inhibition of cell cycle progression. This suggests the possibility of PI 3-kinase 'isoforms', which mediate different intracellular functions. Thus the activation of FRAP or another putative PI 3-kinase isoform may provide one of the links between growth factor receptor activation and p70^{S6k}. Recently, it has been shown that expression of a p85 mutant inhibits insulin-stimulated glucose transport in CHO cells [222], indicating that p85/p110 PI 3-kinase activation is still required for this particular pathway.

Interactions between the Ras/MAP kinase and PI 3-kinase cascades

The strong correlation between growth factor activation of MAP kinase and PI 3-kinase has raised the possibility of direct interactions between the pathways, and indeed a role for p21^{ras} in the activation of PI 3-kinase has been demonstrated recently. Endogenous GTP-bound p21^{ras} is able to bind directly to the 110 kDa catalytic subunit *in vitro* through the Ras effector domain, while in intact cells, expression of dominant negative *ras* mutants or wild-type *ras* results in inhibition or elevation of 3′-phosphorylated phosphoinositide formation respectively [223]. Ras does not seem to play a role in targeting p85 to the receptor for activation since PI 3-kinase activity recovered in phosphotyrosine immunoprecipitates following growth factor stimulation is not affected by overexpression of the negative *ras* mutant N17 [223]. Instead, p21^{ras} may function to specifically target p110 to the plasma membrane or promote the interaction of p110 with its lipid substrate. A recent study has shown that PDGF may activate PI 3-kinase in part by stimulating the relocation of p110 to the receptor and by increasing the intrinsic activity of the enzyme [224], findings consistent with the involvement of additional mechanisms in the regulation of p110.

However, a role for Ras-dependent activation of PI 3-kinase is certainly not apparent in all cell types. Expression of *raf* or *ras* mutants, while inhibiting PDGF-induced activation of MAP kinase, does not affect pp70^{S6k} activation [219]. In the same study it has also been shown that abolition of the kinase insert domain of the PDGF receptor does not affect the activation of p21^{ras} although p70^{S6k} activity is abolished. In addition, it has been shown that insulin-stimulated glucose transport is abolished in CHO cells expressing a PI 3-kinase mutant while activation of p21^{ras} is unaffected [222].

Recent studies have focused upon the interaction between PI 3-kinase and downstream regulators of the MAP kinase cascade and it is possible that PtdInsP_3 may regulate Raf–Ras interaction (see above). PtdInsP_3 may also activate a number of atypical PKC isoforms and therefore the interaction with the MAP

kinase cascade may be indirect [225]. Additional studies using appropriate molecular biological approaches will be required to resolve any intermediate role for PKC in providing a link between the two pathways.

Rho, βγ subunits and G-protein receptor-coupled activation of PI 3-kinase

As with other tyrosine kinase pathways, G-protein-coupled receptor agonists have been shown to stimulate the tyrosine phosphorylation of p85 and activation of PI 3-kinase in a number of cell systems, in particular platelets, neutrophils and fibroblasts [197,215,226]. The involvement of the monomeric low-molecular-mass G-protein, $p21^{rho}$, has again been implicated in some instances. C3 exoenzyme-catalysed ADP ribosylation of Rho prevents guanosine 5′-[γ-thio]triphosphate (GTP[S])-stimulated PI 3-kinase activity in solubilized platelets [215]. The *ras* subfamily member $p21^{rac}$ is unable to activate PI 3-kinase or reverse the effects of C3 exoenzyme, suggesting a specific role for exogenous *rho* in this preparation. Addition of C3 exoenzyme is also able to abrogate LPA-induced tyrosine phosphorylation of PI 3-kinase activity in fibroblasts [197].

In neutrophils and U937 cells, the PI 3-kinase activity stimulated by ATP and the chemotactic peptide formyl-Met-Leu-Phe (fMLP) has been shown to be sensitive to pertussis toxin pretreatment, suggesting the direct involvement of a heterotrimeric G-protein in the pathway [226]. Recently, Stephens and co-workers have identified a PI 3-kinase activity in neutrophils which is activated by βγ subunits [227]. This PI 3-kinase activity has characteristics which distinguish it from the conventional PI 3-kinase activity regulated by receptor tyrosine kinases. A similar activity has also been identified in platelet homogenates which is also believed to be *rho* insensitive [228]. Purification and sequencing of the protein will indicate if this isoform can interact directly with βγ subunits or some other intermediate regulatory protein. In myeloid cells, PI 3-kinase activity is recovered in anti-Src immunoprecipitates following stimulation with granulocyte/macrophage colony stimulating factor [229]. The peptide fMLP also increases the activation of Src kinases; however, the activation of PI 3-kinase by this route does not contribute to the total formation of PtdIns(3,4,5)P_3 stimulated by fMLP [226]. Nevertheless, it is possible that Src may play a role in the coupling of G-protein-linked receptors to PI 3-kinase in other systems since a number of these agonists have been shown to activate c-Src in fibroblasts and other cell types [230].

JAK AND STATS

One of the most recently identified tyrosine kinase signalling cascades involves the activation of members of a novel class of tyrosine kinase, comprising tyrosine kinase 2 (Tyk2) and Janus kinase (JAK), and the tyrosine phosphorylation of cytoplasmic proteins called signal transducers and activators of transcription (STATs). This pathway is activated primarily in response to the α and γ interferons (IFNα and γ) [231,232], IL-3 [233], erythropoietin [234], growth hormone [235] and others. Recently growth factors, in particular EGF [236], have been shown to activate components of this cascade and thus may represent an additional pathway by which some receptor tyrosine kinases can initiate changes in cellular responses.

Members of the JAK/Tyk2 family are characterized by the presence of a second kinase-related domain immediately N-terminal to the tyrosine kinase domain [237–239], but they do not contain SH-2 or SH-3 domains. Nevertheless, cytokine stimulation leads to the association of these kinases with the receptor and their subsequent tyrosine phosphorylation and activation. The activation of individual Tyk2 and JAK proteins is specifically determined by each cytokine; for example JAK-1 and Tyk2 mediate IFNα signalling events while JAK-1 and JAK-2 are involved in responses to IFNγ [239–243]. Other factors such as IL-3 and erythropoietin specifically activate JAK-2 [233,234] while recently it has been shown that IL-2 and IL-4 strongly activate JAK-3 but stimulate JAK-1 to a much lesser extent [244].

Activation of the JAK kinase pathway results in the formation of DNA-binding complexes. This is mediated in part by the tyrosine phosphorylation and activation of STAT proteins. STATs were initially identified as three components, p84, p91 and p113, of the IFN-stimulated gene factor 3 complex (ISGF-3) which was known to mediate the effects of IFNα upon gene expression and long-term cellular responses [245]. The p84 and p91 STATs, encoded by a single gene, contain conserved SH-2 and SH-3 domains [28] and are identical except for a 38-amino-acid N-terminal sequence present in p91, while p113 STAT is approximately 40% homologous with p84 and p91 [245]. Phosphorylation of p91 upon Tyr^{701} is essential for IFN-mediated responses since mutation of this residue prevents p91 translocation to the nucleus and the formation of DNA-binding complexes [243,246]. The specificity in the activation of the Tyk2/JAK cascade by different cytokines outlined above is also reflected in the phosphorylation of different STATs and the regulation of DNA-binding proteins. IFNα activates p81, p91 and p113 and forms the ISGF-3 complex in the nucleus [231]; however, IFNγ primarily activates p91 and stimulates the activation of a different response element, the GAS–GAF complex comprising the gamma-activated site (GAS) and the gamma-activated factor (GAF) [232]. Other cytokines activate different STATs or possibly other unknown intermediates and initiate a unique pattern of transcription events [247,248].

At present it is unclear if the JAK/STAT pathway represents a universal signalling pathway for growth factor receptors. The majority of studies which show growth factor activation of the JAK/STAT kinase cascade utilize epithelial or hepatocyte cell lines which are unusual in a number of respects in terms of their responses to EGF. Nevertheless in these cells, and in some instances in fibroblasts, EGF and also PDGF can stimulate the tyrosine phosphorylation of p91 and its association with DNA-binding elements within the nucleus, including the GAS–GAF complex [249,250]. This response is not dependent upon Ras activation and seems to be activated only by certain growth factors; both FGF and insulin are ineffective despite being able to induce other well-characterized phosphorylation events [250]. Mutation of the EGF receptor has indicated that regions within the receptor normally required for interaction with SH-2 domain-containing proteins are also involved in the activation of p91. In epithelial cells, p91 is recovered from EGF receptor immunoprecipitates [236] but it is at present unknown which specific residues within the EGF receptor are responsible for the direct coupling to p91, if it occurs, or whether the SH-2 domain of p91 is involved in the interaction.

EGF can also stimulate the activation of JAK-1 [243], indicating the potential for the activation of p91 to occur downstream of another receptor kinase interaction. The lack of both SH-2 and SH-3 domains within the structure of JAK-1 makes it likely that an additional intermediate docking protein or an unidentified phosphopeptide sequence is present in the growth factor receptor which can allow specific interaction with JAK. EGF has also been found to activate another member of the STAT family, STAT 3 [247]. However, STAT 3 is not tyrosine phosphorylated by IFNγ and therefore neither JAK-1 nor JAK-2 is likely to be involved in its activation. This suggests the potential of direct

coupling of the EGF receptor to a number of STAT proteins or the involvement of additional JAKs or other tyrosine kinases in the cascade.

SUMMARY

It is apparent that elementary models have now been established outlining the interaction of growth factor and more recently antigen, cytokine and G-protein-coupled receptors with a number of tyrosine kinase pathways such as MAP kinase, PI 3-kinase, pp125^{FAK} and JAK. Although some of these pathways are activated principally by a single class of receptor and a degree of specificity is observed, it is clear that one receptor can usually activate several pathways to different extents. Because of this, it is evident that each receptor cell system will display a unique pattern in terms of the tyrosine kinase pathways activated. The elucidation of more tyrosine kinase cascades suggests that the overall response in terms of tyrosine kinase signals generated will ultimately be unique for each receptor in a given cell system and will give rise to a specific functional outcome. Irrespective of each receptor system studied, in the majority of cases it seems that no single mechanism for the activation of each distinct tyrosine kinase pathway is apparent, but rather several interacting pathways combine to regulate the generation of the tyrosine kinase signals.

FUTURE PROSPECTS

Translocation of tyrosine kinase signalling molecules to activated receptors

An obvious and outstanding question regarding the activation of tyrosine kinase pathways involves the spatio-temporal aspects of pathway activation. As a large number of tyrosine kinases or associated intermediates are either cytosolic or not associated with the receptor before agonist activation, then a key question relates to the processes which regulate the translocation of intermediates to the receptor itself. Recent evidence suggests that the activation of Grb2/mSos may only require the recruitment of Grb2 to the plasma membrane without association with the receptor. Although it is already recognized that for Grb2 and other non-receptor-associated proteins such as ZAP-70 and p72^{syk}, the presence of intact SH domains are required for translocation, the mechanisms underlying this process have not been elucidated. This is of particular importance for the antigen and cytokine receptors where association with non-receptor tyrosine kinases is a requisite for full receptor function as well as optimum activation of the signalling cascades. It is also likely that translocation will be a feature of G-protein-coupled receptor activation of tyrosine kinase cascades directly, and the Src kinases are obvious candidates for this role. Finally, the specificity of the translocation process may represent an additional mechanism in ensuring an element of fidelity in the activation of each pathway. Given the multitude of other tyrosine kinases such as pp125^{FAK}, pp60^{src} and JAK that can be associated with different compartments of the cell following activation, it is clear that there is much to learn of the spatio-temporal aspects of tyrosine kinase signalling.

Defining the MAP, MEK and SAP kinase pathways: the search for another MEK kinase?

While the initial elucidation of the Ras/MAP kinase cascade was a major achievement in defining growth factor and G-protein-receptor-coupled tyrosine kinase pathways, the idea of a single pathway resulting in the activation of MAP kinase is rapidly becoming conceptually obsolete. The discovery of MEK kinase and the SAP kinase cascade indicates the potential for multiple variations in the MAP kinase paradigm. Much of the future work will be aimed at dissecting out a precise role of proteins within these cascades and their degree of interaction with other kinases such as the ceramide-activated kinases and isoforms of PKC as well as with immediate products of the phospholipid signalling cascades.

Two immediate questions still remain to be answered. What mechanisms regulate the activation of Raf-1, and what is the role of the 14-3-3 proteins? Secondly, is MEK kinase a component of the MAP or SAP kinase pathway? If MEK kinase is indeed upstream of the stress-activated SEK-1 and not MEK, this still implicates the involvement of another putative MEK kinase which is distinct from Raf and which functions as a component of a growth factor-activated MAP kinase cascade.

We regret that due to considerations of space we are unable to quote all the literature relevant to this review. We would like to thank Diane Dixon and G. W. Gould for critical reading of the manuscript. We also thank G. W. Gould for his advice and encouragement over the past three years. Work quoted from R. P.'s laboratory is supported by grants from the Medical Research Council, National Asthma Campaign, British Heart Foundation and The Wellcome Trust.

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